Phenformin Inhibits Myeloid-Derived Suppressor Cells and Enhances the Anti-Tumor Activity of PD-1 Blockade in Melanoma

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Biguanides, such as the diabetes therapeutics metformin and phenformin, have shown antitumor activity both in vitro and in vivo. However, their potential effects on the tumor microenvironment are largely unknown. Here we report that phenformin selectively inhibits granulocytic myeloid-derived suppressor cells in spleens of tumor-bearing mice and ex vivo. Phenformin induces production of reactive oxygen species in granulocytic myeloid-derived suppressor cells, whereas the antioxidant N-acetylcysteine attenuates the inhibitory effects of phenformin. Co-treatment of phenformin enhances the effect of anti-PD-1 antibody therapy on inhibiting tumor growth in the BRAF V600E/PTEN-null melanoma mouse model. Combination of phenformin and anti-PD-1 cooperatively induces CD8+ T-cell infiltration and decreases levels of proteins that are critical for immune suppressive activities of myeloid-derived suppressor cells. Our findings show a selective, inhibitory effect of phenformin on granulocytic myeloid-derived suppressor cell-driven immune suppression and support that phenformin improves the anti-tumor activity of PD-1 blockade immunotherapy in melanoma.

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are one of the major types of immune cells that contribute to tumor-induced immune suppression and escape from immune elimination (Marvel and Gabrilovich, 2015). They can be broadly divided into two main populations, granulocytic (G-) (also known as polymorphonuclear) and monocytic (M-) MDSCs, which are characterized by CD11b+Ly6GhiLy6Cint and CD11b+Ly6Glo-Ly6Cint, respectively, in mouse models (Marvel and Gabrilovich, 2015). MDSCs exert potent immunosuppressive activities toward T cells through multiple mechanisms, including expression of arginase 1, inducible nitric oxide synthase, and release of reactive oxygen species (ROS) (Marvel and Gabrilovich, 2015). Given their prominent role in tumor immune evasion, targeting MDSC-mediated immune suppression has been suggested to be an attractive approach to modulate tumor immunity for treating cancers (Draghiciu et al., 2015; Marvel and Gabrilovich, 2015).

Biguanides, such as metformin and phenformin, are commonly used to treat type 2 diabetes. Treatment with metformin in patients with type 2 diabetes has been found to be associated with lower cancer risks and lower cancer-related mortality rates (Morales and Morris, 2015). Biguanides inhibit mitochondrial complex 1 of the respiratory chain, which increases the adenosine monophosphate (AMP) to adenosine triphosphate ratio, resulting in AMP-activated protein kinase (AMPK) activation, which has been proposed to be a major mediator of their anti-tumor activity (Hardie et al., 2015; Pollak, 2013; Wheaton et al., 2014). However, most studies on the anti-tumor activities of biguanides have been focused on their cell-autonomous effects on cancer cells, but their potential effects on immune cells within the host and tumor microenvironment have remained largely unexplored.

We previously investigated the anti-tumor effect of biguanide AMPK activators in combination with BRAF inhibitors in various preclinical melanoma models (Shen et al., 2013; Zheng et al., 2009). These studies showed that phenformin, but not metformin, enhanced the efficacy of BRAF inhibitors by inhibiting the proliferation of BRAF-mutated melanoma cells in vitro and suppressing BRAF-driven tumor growth in mouse models (Yuan et al., 2013). More recently, we also found that phenformin and the extracellular signal-regulated kinase inhibitor SCH772984 exhibited synergistic anti-proliferative activities in NF1-mutant melanoma cells (Trousil et al., 2014). However, their potential effects on the tumor microenvironment are largely unknown. Here we report that phenformin selectively inhibits granulocytic myeloid-derived suppressor cells in spleens of tumor-bearing mice and ex vivo. Phenformin induces production of reactive oxygen species in granulocytic myeloid-derived suppressor cells, whereas the antioxidant N-acetylcysteine attenuates the inhibitory effects of phenformin. Co-treatment of phenformin enhances the effect of anti-PD-1 antibody therapy on inhibiting tumor growth in the BRAF V600E/PTEN-null melanoma mouse model. Combination of phenformin and anti-PD-1 cooperatively induces CD8+ T-cell infiltration and decreases levels of proteins that are critical for immune suppressive activities of myeloid-derived suppressor cells. Our findings show a selective, inhibitory effect of phenformin on granulocytic myeloid-derived suppressor cell-driven immune suppression and support that phenformin improves the anti-tumor activity of PD-1 blockade immunotherapy in melanoma.

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMP, adenosine monophosphate; AMPK, adenosine monophosphate-activated protein kinase; CFSE, carboxyfluorescein succinimidyl ester; G-MDSC, granulocytic myeloid-derived suppressor cell; MDSC, myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; ROS, reactive oxygen species

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2017). In this study, we examine the effects of phenformin on immune cells in the tumor microenvironment. We found that phenformin selectively inhibits G-MDSCs in vivo and in vitro. More importantly, we found that treatment with phenformin enhances the effect of anti-PD-1 immune checkpoint blockade in a genetically engineered BrafV600E/PTennull (BRAF/PTEN-) driven mouse model of melanoma. These findings provide a rational basis for future clinical evaluation of phenformin/anti-PD-1 combination therapy.

**RESULTS**

We first determined whether phenformin and metformin modulate MDSC accumulation in the spleens of tumor-bearing mice. Splenocytes from immune competent FVB mice harboring BP01 mouse melanoma allografts were harvested and evaluated by flow cytometry for the proportion of G-MDSCs (CD11b+Ly6GhiLy6Chi) and M-MDSCs (CD11b+Ly6GhlLy6Ch) (Figure 1a). We found a significant decrease of G-MDSCs, but not M-MDSCs, in the spleens of phenformin-treated mice compared with those treated with either metformin or the vehicle control group (Figure 1b and c). Neither phenformin nor metformin significantly changed the proportions of total macrophages, M2 macrophages, CD4+ T cells, CD8+ T cells, or regulatory T cells in the spleens of these mice (see Supplementary Figure S1a—e online). These results support that treatment by phenformin, but not metformin, selectively reduces the proportion of G-MDSCs in the spleens of tumor-bearing mice. We previously observed similar differential effects of phenformin and metformin on inhibiting melanoma cell proliferation, and the difference was shown to be attributable to the low expression levels of OCT2 in melanoma cells, which is required for uptake of metformin, but not the more lipophilic phenformin (Yuan et al., 2013). Similarly, we found that OCT2 expresses upon treatment of phenformin (see Supplementary Figure S1f). These results together suggest that, in addition to reducing the number of G-MDSCs, phenformin may also impair the immune-suppressive function of M-MDSCs than of G-MDSCs (Movahedi et al., 2008). Consistent with a role of phenformin in increasing ROS levels in G-MDSCs, we found that the mRNA expression of a major antioxidant enzyme, nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase 1, dropped remarkably upon treatment of phenformin (see Supplementary Figure S3e). Moreover, we found that phenformin also decreased the amount of CHOP, a stress sensor protein that is critical for the activity of MDSCs (Thevenot et al., 2014) (see Supplementary Figure S3f). These results together suggest that, in addition to reducing the number of G-MDSCs, phenformin may also impair the immune-suppressive function of MDSCs. Future studies will be carried out to further investigate the molecular mechanisms underlying the inhibitory effects of phenformin on G-MDSCs.

Therapeutic immune checkpoint blockade that targets cell surface inhibitory checkpoint regulators of T-cell activation, such as CTLA-4 and PD-1, has shown significant clinical benefits in a subset of melanoma patients (Sharma and Allison, 2015). Although most of the clinical responses to these therapies appear durable, the overall response rate remains low. Given the inhibitory effects of phenformin on immune-suppressive MDSCs, we sought to test whether phenformin may enhance the efficacy of anti-PD-1 checkpoint blockade using the BRAF/PTEN genetically engineered mouse model. We found that treatment with anti-PD1 antibody or phenformin alone only slightly inhibited tumor growth compared with IgG isotype control (Figure 3a—c). However, the combination of both phenformin and anti-PD1 antibody significantly inhibited tumor growth (Figure 3b and c). Unlike phenformin, we found that metformin had minimal effects by itself or in combination with anti-PD-1 antibody in this tumor model (see Supplementary Figure S4a and b online). Phenformin alone or in combination with anti-PD-1 significantly decreased the proportion of G-MDSCs, but not M-MDSCs, within the tumor and spleen (Figure 4a—d).
Anti-PD-1 antibody did not significantly affect G-MDSC numbers by itself and did not further modulate the effect of phenformin seen on this population (Figure 4a–d, and see Supplementary Figure S5a online). We also did not observe significant effects of these treatments on the numbers of natural killer cells in tumors or macrophages or regulatory T cells in spleens of these mice (see Supplementary Figure S5b–e). Consistent with the flow cytometry results, immunofluorescence analysis indicated that the number of cells that were co-stained with both CD11b and Gr1 (Ly-6G/Ly-6C) antibodies decreased similarly in tumors treated with phenformin alone or combined with anti-PD-1, compared with control (Figure 5a, and see Supplementary Figure S6 online). Tumors treated with the combination of phenformin and anti-PD-1 expressed significantly less arginase 1, S100A8, and S100A9 mRNA, which are key mediators of MDSC-driven immune suppression (Figure 6a–d). Immunohistochemical analysis confirmed that the protein levels of arginase 1 were also reduced in tumor specimens treated with phenformin in combination with anti-PD-1 (Figure 6b). Together, these results suggest that phenformin reduces the numbers of G-MDSCs and may inhibit their immune suppressive activities toward CD8\(^+\) T cells and that phenformin and PD1 blockade may cooperate to induce anti-tumor responses and increased CD8\(^+\) T-cell infiltration into the tumor bed.

**DISCUSSION**

Our current study shows that, in addition to tumor-intrinsic effects, phenformin also exerts anti-tumor activities by countering the immunosuppressive activity of MDSCs, which promote tumor development and immune evasion. Phenformin and its analog metformin belong to the biguanide class of anti-diabetes drugs. Several epidemiological studies have found that treatment of metformin in patients with type 2 diabetes is associated with lower cancer risk and lower cancer-related mortality rates when compared with other diabetes therapeutics (Morales and Morris, 2015). Significantly, numerous preclinical studies in tumor cell culture, xenograft, carcinogen-induced, and genetically modified
mouse models have shown direct anti-tumor activities for both metformin and phenformin (Pollak, 2013; Wheaton et al., 2014). Effects of metformin on T cells have recently been reported in tumor-bearing mice. Metformin was shown to promote CD8\(^+\) memory T-cell generation and protective immunity (Pearce et al., 2009). Loss of AMPK\(\alpha_1\) in T cells impaired the development of T helper type 1 and T helper type 17 cells in mice (Blagih et al., 2015). More recently, metformin was found to increase the ratio of effector memory T cells to central memory T cells, enhance tumor infiltration of CD8\(^+\) T cells, and protect them from apoptosis in the tumor microenvironment (Eikawa et al., 2015). In the BRAF/PTEN melanoma model, we observed a significant change of CD8\(^+\) T-cell tumor infiltration only in the mice treated with both phenformin and anti-PD-1 antibody (Figure 6e). However, phenformin by itself did not appear to affect tumor infiltration of cytotoxic T cells (Figure 6e) or the numbers of CD4\(^+\) or CD8\(^+\) T cells in the spleens of tumor-bearing mice (see Supplementary Figure 1d and e), suggesting that phenformin does not affect T-cell function in these mouse models.
In addition, our data support that phenformin, but not metformin, decreased the number of MDSCs in tumor-bearing mice. This difference in the effects of these two biguanides may be due to the low expression levels of OCT2 in MDSCs (Yuan et al., 2013). We also found that phenformin promotes BRAF inhibitor anti-tumor efficacy in the immune-competent BRAF/PTEN-driven melanoma model (Yuan et al., 2013). It will therefore be interesting to investigate whether the increased anti-tumor activity observed for BRAF inhibitors in combination with phenformin might be dependent on the inhibitory effects of phenformin on MDSCs.

Biologic drugs that target the immune checkpoint proteins CTLA-4, PD-1, or PD-L1 have shown significant clinical benefits in solid tumors with durable responses but only in a fraction of patients (Sharma and Allison, 2015). Therefore, improving the response rates of these immune checkpoint blockade therapies represents a major challenge in cancer treatment. In addition to using immune checkpoint pathways to evade immune surveillance, tumor cells also promote the expansion and activation of cellular populations that act to suppress the function of cytotoxic immune cells, such as MDSCs. Indeed, MDSCs have been proposed to contribute to resistance to various cancer therapies, including to anti-CTLA-4 and anti-PD-1 blockade (Martens et al., 2016; Sade-Feldman et al., 2016; Weber et al., 2016). Hence, targeting MDSCs represents an attractive approach to modulating tumor immunity for treating cancers. We have shown here that phenformin decreased the proportion of G-MDSCs in both tumors and spleens in the BRAF/PTEN mouse melanoma model and enhanced the effect of anti-PD-1 antibody on inhibiting tumor growth; thus, targeting MDSCs may be synergistic with immune checkpoint blockade. Because our data suggest that phenformin selectively targets G-MDSCs but not M-MDSCs, it is plausible that M-MDSCs remain functional and exert their suppressive activity on T cells in this model and that further depletion of M-MDSCs may achieve even better anti-tumor response. The translational impact of phenformin/anti-PD-1 combination could potentially be extended from melanoma to other solid tumors, especially in those tumors where G-MDSCs contribute to tumor immune evasion and tumor maintenance. Conceptually, given this effect on G-MDSC function, other immune therapeutic approaches may also benefit from the addition of phenformin in addition to checkpoint blockade. In addition, such perturbation of MDSC function by phenformin may improve conventional chemoradiation regimens, the efficacy of which depend on the stimulation of immune effector functions against the tumor.

Finally, because of rare cases of lactic acidosis, (although this complication is less common with metformin compared with phenformin), phenformin was discontinued for the treatment of diabetes by the US Food and Drug Administration in the late 1970s, but it is being used in some other countries. The rare complication of lactic acidosis of phenformin is minor compared with the toxicities of commonly used cancer chemotherapy, and therefore clinical evaluation to repurpose phenformin for cancer treatment is warranted in light of recent preclinical studies on phenformin in cancer (Shackelford et al., 2013; Trousil et al., 2017; Yuan et al., 2013). A phase I clinical trial for phenformin in combination with dabrafenib and trametinib has recently been initiated (NCT03026517). This study provides a rational basis for future clinical evaluation of phenformin in combination with anti-PD-1 therapy.

MATERIALS AND METHODS

Animal studies
All animal experiments were conducted in accordance to the Institutional Animal Care and Use Committee guidelines at the...
Massachusetts General Hospital. For allograft models, 6-week-old female FVB mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were injected subcutaneously in the right lateral flank with BP01 mouse melanoma cells that were originally derived from BRAFCA/+PTENlox/lox murine melanoma (Yuan et al., 2013). Once tumor sizes reached 120–180 mm³, animals were randomly assigned to three groups that were administered water, phenformin (100 mg/kg/day), or metformin (300 mg/kg/day) in the drinking water for 10 days. For the BRAF/PTEN genetically engineered mouse model, 6- to 10-week-old Tyr::CreER; BrafCA/+; Ptenlox/lox mice were originally acquired from M. McMahon and M.W. Bosenberg (Dankort et al., 2009). Mice were topically administrated 5 mmol/L 4-hydroxytamoxifen (H6278, Sigma-Aldrich, St. Louis, MO) to induce tumor formation. Once tumor sizes reached 120–180 mm³, animals were randomly assigned to four groups and administered 100 μg of rat IgG2a isotype control or rat anti-PD-1 antibody (LEAF Purified, Biolegend, San Diego, CA) (antibodies used in this study are listed in Supplementary Table S1 online) by intraperitoneal injections, phenformin (100 mg/ kg/day) together with IgG isotype control, or phenformin together with anti-PD-1 antibody (same dose as in single-agent groups). Antibodies were administered every other day until day 5 for immunophenotyping by flow cytometry (Figure 1), until day 13 for experiments monitoring tumor growth (Figure 3), or until day 7 to assess tumor-infiltration of MDSCs (Figure 4). Tumor dimensions were calculated from caliper measurements by using the following formula: \((D \times d)/2\), where \(D\) represents the large diameter and \(d\) the small diameter of the tumor.

**Flow cytometry**

Single-cell suspensions were prepared from spleens by mechanical dissociation, followed by removal of red blood cells with

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**Figure 4. Phenformin decreases the number of G-MDSCs in the BRAF/PTEN melanoma mouse model. (a–d)** Tumors and spleens were harvested 7 days after treatment and analyzed for G-MDSCs and M-MDSCs in single-cell suspension of BRAF/PTEN mouse tumors and spleens from different treatment groups as shown in Figure 3. Nine to 12 mice per group were analyzed from three independent experiments. Individual data and mean are shown. *\(P < 0.05\); **\(P < 0.01\); ns, not significant (nonparametric Mann-Whitney test). Ctrl, control; G-MDSC, granulocytic myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; Phen, phenformin.

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**Figure 5. Effects of the combination of phenformin and anti-PD-1 on CD11b and Gr1 double positive cells and arginase 1-expressing cells in the BRAF/PTEN mouse model. (a–b)** Tumor samples from various treatment groups shown in Figure 3 were stained for CD11b (red) and Gr1 (green) by (a) immunofluorescence and (b) arginase 1 by immunohistochemistry. Ctrl, control; Phen, phenformin. Scale bar = 50 μm.
ammonium-chloride-potassium lysing buffer (Lonza, Basel, Switzerland). Tumors were shredded into small pieces and incubated for 45 minutes at 37°C in collagenase-containing buffer comprised of 100 U/ml of collagenase type IV (Worthington, Lakewood, NJ), 50 mg/ml of DNase I (Worthington), and 10% fetal bovine serum in RPMI-1640 medium (Life Tech, Carlsbad, CA). After incubation, cells were treated with ammonium-chloride-potassium lysing buffer and passed through a 70-μm cell strainer to remove debris. All samples were washed and resuspended in FACS buffer (phosphate buffered saline with 2% fetal bovine serum). Single-cell suspensions were incubated with anti-CD16/CD32 mAb (eBioscience, San Diego, CA) to block FcγRII/III receptors for 10 minutes on ice and stained for 30 minutes with various fluorochrome-conjugated monoclonal antibodies (see Supplementary Table S1). Cells were then washed and resuspended in 7-AAD viability staining solution (Biolegend). Dead cells and doublets were excluded on the basis of forward and side scatter. For staining of intracellular antigens, cells were permeabilized using Foxp3 Fixation and Permeabilization Kit (eBioscience) before staining. Cells were acquired on a Canto Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were collected for 10^5–10^6 cells and analyzed with FlowJo software (TreeStar, Ashland, OR).

**BM-derived MDSCs**
A Transwell system (Corning, Corning, NY) was used for co-culture of BM cells with BP01 tumor cells. Briefly, BM cells were obtained from the femurs and tibias of naïve FVB mice and plated at 2 × 10^5 cells/ml in MDSC medium on the lower chamber of the Transwell system. MDSC medium composed of RPMI 1640, 10% fetal bovine serum, 50 mM 2-mercaptoethanol, 10 mM HEPES buffer, 5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. BP01 cells were seeded in the upper chamber of Transwell system (0.4 μm pore size, Corning). Cultures were incubated at 37°C and 5% CO2 for 3 days. On day 3 of culture, the upper chamber containing BP01 cells was removed, and fresh medium containing cytokines IL-6, G-CSF, and GM-CSF (all at 40 ng/ml and from Peprotech, Rocky Hill, NJ) was added to the lower chamber. Phenformin (1 mM), metformin (3 mM), AICAR (1 mM), or Compound C (20 μM; Calbiochem, Darmstadt,

**Figure 6. Phenformin modulates the immune suppressive function of MDSCs in the BRAF/PTEN melanoma mouse model.** (a–d) Tumors were harvested on day 7 after treatment as shown in Figure 3 and mRNA levels of arginase 1, S100A8, S100A9, and iNOS were quantified by quantitative PCR (n = 9–10). (e) Tumor-infiltrating T cells (CD45^+CD3^+CD8^+^) were analyzed by flow cytometry. Tumors of BRAF/PTEN mice were harvested 7 days after various treatments. Twelve to 16 mice per group from three independent experiments were analyzed. Individual data and mean are shown; *P < 0.05 (nonparametric Mann-Whitney test). (f) CFSE-labeled CD8^+^ T cells activated with anti-CD3/CD28 and co-cultured at the different ratios (1:1/1:2/1:4) with splenic MDSCs sorted from control, phenformin, or metformin treated BP01 tumor-bearing mice for 10 days. T-cell proliferation was monitored 72 hours after co-culture. Results are expressed as mean ± standard error of the mean from three independent experiments. *P < 0.05. ns, not significant (Student t test). Arg-1, arginase 1; Ctrl, control; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cell; Phen, phenformin.
Germany) was added into the lower chamber for 24 hours, and the proportion of G-MDSCs and M-MDSCs was assessed by flow cytometry. Phenformin, metformin, and AICAR were purchased from Toronto Research Chemicals (Toronto, Canada).

**ROS measurement**

BM-MDSCs were induced with tumor-conditioned medium for 3 days. Subconfluent BP01 tumor cells were kept in RPMI medium with reduced serum concentration (3%) for 48 hours before supernatants were collected and filtered (0.22 μm). BM cells from FVB naïve mice were cultured in MDSC medium in presence of 30% (volume/volume) BP01-conditioned medium for 72 hours to induce BM-MDSCs. For ROS measurement, BM-MDSCs were treated with phenformin (1 mmol/L), N-acetyl-L-cysteine (10 mmol/L, Sigma) for 24 hours before incubation with 2.5 μmol/L dichlorodihydrofluorescein diacetate or 5 μmol/L CellROX Deep Red Reagent (Invitrogen, Carlsbad, CA) in RPMI 1640 medium at 37 °C for 30 minutes. Cells were then labeled with anti-CD11b-APC, anti-Ly-6C-PE-Cy7, and anti-Ly-6G-APC-Cy7 antibodies for 20 minutes, followed by flow cytometry analyses.

**Splenic G-MDSCs**

Splenic G-MDSCs were positively selected with Ly-6G antibody by magnetic-activated cell sorting (MDSC isolation kit; Miltenyi Biotech, Cambridge, MA). Purified cells were routinely more than 90% CD11b^+Ly6G^+ as confirmed by flow cytometry. Splenic G-MDSCs were cultured in MDSC medium and treated with phenformin (1 mmol/L) or metformin (3 mmol/L) for 48 hours. BrdU (10 μmol/L) was added 4 hours before harvesting. After fixation and permeabilization using Foxp3 Fixation and Permeabilization Kit (eBioscience), cells were stained with mouse anti-BrdU antibody. Alexa 488-conjugated anti-mouse IgG was used as secondary antibody. The percentage of BrdU^+ cells was measured by flow cytometry to evaluate proliferation. Annexin V staining was performed in Annexin V binding buffer (BD Biosciences) according to the manufacturer’s recommendations. Apoptosis was analyzed based on the percentage of Annexin V^+ cells in splenic G-MDSCs after 24 hours of drug treatment.

**Real-time PCR**

Total RNA was extracted using the RNaseasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, and cDNAs were prepared using the RevertAid Reverse Transcription Kit (Thermo Fisher Scientific, Cambridge, MA). Quantitative PCR was performed using the SYBR Green I Master (Roche, Basel, Switzerland) reaction mix on a Light Cycler 480 (Roche). Each sample was tested in triplicate, and results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase or 36B4 as an internal control. Relative gene expression was calculated by the 2^ΔΔCt method. Specific primer sequences used in this study are listed in Supplementary Table S2 online.

**Immunohistochemistry and immunofluorescence**

Mouse tissue sections were prepared for immunohistochemistry and immunofluorescence as previously described (Yuan et al., 2013).

**T-cell suppression assay**

Splenocytes from FVB naïve mice, depleted of red blood cells, were stained with anti-CD8-FITC. CD8^+ T cells were purified using anti-FITC-microbeads (Miltenyi Biotech) according to the manufacturer’s protocol and labeled with 1 μmol/L CFSE (Invitrogen) in phosphate buffered saline for 10 minutes at 37 °C. Purity was confirmed to be over 90% by flow cytometry. CD8^+ T cells were placed in triplicate into U-bottom 96-well plates (2 × 10^5/well) coated with 10 μg/ml anti-CD3 antibody and 0.5 μg/ml anti-CD28 antibody. Purified Ly-6G^+ G-MDSCs from spleens of BP01 tumor-bearing FVB mice were incubated with CD8^+ T cells at 37 °C for 72 hours. On day 3, cells were stained with anti-CD8-APC and analyzed for CFSE dilution by flow cytometry to measure proliferation of CD8^+ T cells.

**Statistical analysis**

Statistical evaluations were conducted using two-tailed Student t test or Mann-Whitney U test. Appropriate statistical corrections were used when normal distribution were not given. Statistical analyses were performed using GraphPad Prism (La Jolla, CA). P values less than 0.05 were considered statistically significant.

**CONFLICT OF INTEREST**

BZ serves on the Scientific Advisory Board of Enlibrium, a company developing drugs that target cancer metabolism. The authors state no other conflicts of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.03.033.

**REFERENCES**


